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Fungal Communities Associated with the Biodegradation of Polyester Polyurethane Buried under Compost at Different Temperatures

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Plastics play an essential role in the modern world due to their low cost and durability. However, accumulation of plastic waste in the environment causes wide-scale pollution with long-lasting effects, making plastic waste management expensive and problematic. Polyurethanes (PUs) are heteropolymers that made up ca. 7% of the total plastic production in Europe in 2011. Polyester PUs in particular have been extensively reported as susceptible to microbial biodegradation in the environment, particularly by fungi. In this study, we investigated the impact of composting on PUs, as composting is a microbially rich process that is increasingly being used for the processing of green waste and food waste as an economically viable alternative to landfill disposal. PU coupons were incubated for 12 weeks in fresh compost at 25°C, 45°C, and 50°C to emulate the thermophilic and maturation stages of the composting process. Incubation at all temperatures caused significant physical deterioration of the polyester PU coupons and was associated with extensive fungal colonization. Terminal restriction fragment length polymorphism (TRFLP) analysis and pyrosequencing of the fungal communities on the PU surface and in the surrounding compost revealed that the population on the surface of PU was different from the surrounding compost community, suggesting enrichment and selection. The most dominant fungi identified from the surfaces of PU coupons by pyrosequencing was *Fusarium solani* at 25°C, while at both 45°C and 50°C, *Candida ethanolica* was the dominant species. The results of this preliminary study suggest that the composting process has the potential to biodegrade PU waste if optimized further in the future.

Polyurethanes (PUs) are synthetic plastics with a wide range of applications in the medical, automotive, construction, furnishing, and industrial sectors (1, 2). Polyurethanes are formed by the condensation of polyisocyanate and polyols and are heteropolymers (3). Seven percent of the total plastics manufactured in Europe (47 Mtonnes) in 2011 were polyurethanes (4). A large proportion of plastic waste is directed to landfill sites; however, their low degradation rates, scarcity of landfill sites, and growing water and land pollution problems require the development of alternative waste management strategies (5, 6). Polyester PUs in particular are known to be vulnerable to microbial attack, as they contain ester and urethane linkages that are naturally vulnerable to enzymatic degradation (7). In contrast, polyether PUs, which contain ether linkages within the polymer backbone, are reported to be far more recalcitrant (7, 8).

The development of large-scale commercial composting facilities for the treatment of green and food waste has been helpful in reducing landfill and in meeting recycling goals (9). Composting is a managed self-heated, aerobic process that controls the biological decomposition and transformation of biodegradable materials into a humus-like substance (compost). It is a natural process that results in the production of CO_2, H_2O, minerals, and stabilized organic matter (10, 11). The major advantages of composting are that it is rapid, relatively inexpensive, and environmentally friendly. It is a natural degradation process similar to degradation in the soil, but during composting, a considerable amount of heat is produced as a result of microbial respiration that accelerates the rate of deterioration (12). Thus, temperature is the most important factor affecting the growth and activity of the microbial population (10). Microbiological development progresses in defined temperature phases. During the initial phase of composting, the temperature rises from the ambient temperature to 45°C. As the temperature continues to increase, the composting process enters the thermophilic phase and is dominated by thermophilic microbes with temperatures increasing to 70°C or higher. Once readily metabolized substrates have been utilized, microbial activity and temperature decrease, and eventually the temperature approaches the ambient temperature (maturation phase) (13). Extreme changes in temperature make composting an ecologically complex system, and distinct diverse microbial populations are present at different phases (14).

Previously, fungi have been shown to be the dominant microorganisms involved in the biodegradation of polyester PU when buried in soil (15–17), and a number of fungal species that are capable of degrading PU have been isolated and identified (7, 15–20). In this study, we investigated the potential of the composting process to deteriorate PU by comparing the rate of biodegradation when buried in compost at different temperatures representing the mesophilic and thermophilic stages. We report that polyester PU undergoes significant degradation under composting conditions, while polyether PU appeared largely unaffected. The results indicate that composting has the potential to be developed as a potential alternative waste management route for polyester PU.

MATERIALS AND METHODS

Fabrication of PU coupons. Polyester and polyether polyurethane (PU) coupons of 16 by 16 by 0.1 cm (total surface area of 518.4 cm^2) were fabricated from PU beads (Ellastollan 685 A10 and Ellastollan 1180 A10, respectively) by melting at 180°C at 8-bar pressure in a compression molding machine (Moore, Birmingham, United Kingdom). Dumbbells

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(total length of 5 cm, width at the end of 1.9 cm with 19-cm gauge length) were cut from the coupons using a molder cutter (Wallace Instruments, United Kingdom). Rectangular coupons 4 by 4 cm were cut using a scalpel blade.

Burial of polyurethane coupons in compost and soil. Fresh mature compost (temperature ca. 65°C) (The Compost Shop, United Kingdom) and soil (Online Turf, Lancashire, United Kingdom) were sieved through a 4-mm mesh prior to use. The percent water-holding capacity (WHC) and moisture content (21) of compost and soil were determined, and moisture content was adjusted to 40% by the addition of sterile distilled water.

Airtight plastic containers (25 by 20 by 10 cm) were rinsed in 70% (vol/vol) ethanol, air dried, and filled to 2/3 height with compost or soil. Holes were pierced in the lids of the containers and covered with parafilm to allow gaseous exchange. PU samples (dumbbells and rectangular coupons) were weighed and buried vertically 1 cm apart so that the tops of the coupons were approximately 3 cm below the surface, and the containers were incubated at 25°C, 45°C, or 50°C for up to 12 weeks. The moisture content of the compost and soil was monitored periodically by weighing the containers every 2 days, and the moisture content was maintained at 37 to 40% by the addition of sterile water using a plant spray. Compost and soil samples (10 g) were taken for community analysis prior to incubation (day 0) and periodically after incubation at the three different temperatures.

Recovery of polyurethane samples and enumeration of fungi. Polyurethane samples were recovered from the compost or soil using forceps, and biomass was recovered from the rectangular coupons by the method of Cosgrove et al. (17). An aliquot of 1 ml was used to enumerate the total fungal viable count by plating onto compost extract agar (15) containing 50 μg/ml chloramphenicol (to suppress bacterial growth) following serial dilution in phosphate-buffered saline. PU-degrading microbes were enumerated by plating onto polyurethane agar (PUA) (22) supplemented with chloramphenicol (50 μg/ml) and containing 0.75% (vol/vol) impranil (a liquid dispersion of PU; Bayer, Newbury, United Kingdom) as the sole carbon source. The plates were incubated for up to 1 week at the same temperature the PU samples had been incubated. As fungal colonies on PUA were difficult to differentiate morphologically, individual colonies were transferred onto potato dextrose agar (PDA) (Oxoid UK) and incubated for up to 1 week at the same temperature the PU samples had been incubated. Their morphotypes were determined, and the colonies were grouped into different morphotypes.

Microscopic analysis of PU coupons. Coupons recovered after 12 weeks were washed with sterile distilled water and then with 70% (vol/vol) ethanol, air dried, and observed by environmental scanning microscopy (ESEM) (FEI Quanta 2000 Netherlands) to visualize the surfaces of the coupons. ESEM examination of coupons was done at low pressure (torrs) and room temperature with distance covered from 1,000- to 5-μm scale range.

Tensile strength and weight loss determination. The tensile strength of PU dumbbells was determined using a Tinius Ohlsen H5KT-0586 (United Kingdom) with cross head speed of 1.5 cm/min. Tensile strength was measured as the maximum load (*N*) required to break PU dumbbells. In addition, the weight of PU dumbbells was determined after washing them thoroughly in distilled water. In addition to unburied controls, PU coupons were also autoclaved at 121°C for 5 min and completely immersed in filter-sterilized (0.22-μm) compost or soil extract (23) in sterile 15-ml tubes and incubated at 25°C, 45°C, and 50°C for 12 weeks to observe the effect of hydrolysis.

Extraction, amplification, and purification of genomic DNA from the isolated PU-degrading fungal colonies. Genomic DNA was extracted from the mycelium of fungal colonies by the method of Feng et al. (24). Mycelium/spores (ca. 20 mg) were collected from the surfaces of confluent PDA cultures with a sterile toothpick and placed into 1.5-ml centrifuge tubes containing 0.5 g of 0.5-mm-diameter glass beads. After the addition of 0.65 ml lysis buffer (100 mM Tris-HCl [pH 8.0], 50 mM EDTA [pH 8.0], 1% [wt/vol] SDS, 10 μg ml^{-1} RNase A), the contents of the tubes were homogenized twice for 30 s each time and centrifuged for 2 min at 13,000 rpm. After centrifugation, 500 μl of supernatant was transferred into a new tube containing 100 μl of potassium acetate buffer (3.0 M, pH 5.5). The tube was inverted several times and centrifuged for 2 min at 13,000 rpm. Five hundred microliters of supernatant was transferred into a new tube containing 500 μl of isopropanol, inverted several times, and centrifuged for 2 min at 13,000 rpm. The supernatant was removed, and the DNA pellet was washed with 750 μl of 70% (vol/vol) ethanol. After centrifugation for 30 s, ethanol was removed, and the DNA pellet was air dried for 5 to 10 min. DNA was dissolved in 50 μl sterile distilled water and stored at −20°C until required.

Isolates were identified by the method of Webb et al. (25) using the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) amplified using the fungal universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (26). The reaction mixture contained genomic DNA (20 to 100 ng), primers (2 μM), $MgCl_2$ (1.5 mM), 1× NH_4 reaction buffer (Bioline UK), 200 μM each of deoxynucleoside triphosphates (dNTPs), and 1 U *Taq* polymerase (Bioline UK). The PCR consisted of an initial denaturation at 94°C for 3 min and then 35 cycles, with 1 cycle consisting of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 3 min. PCR amplicons varied between ca. 500 to 575 bp depending on the species. Amplified PCR products were visualized by gel electrophoresis (1% [wt/vol] agarose), and PCR products were purified using the QIAquick PCR purification kit (Qiagen UK) according to the manufacturer's instructions.

ITS rDNA sequencing and identification. Purified samples were sequenced in-house (Faculty of Life Sciences, University of Manchester, United Kingdom) using an ABI Prism 3100 genetic analyzer (Applied Biosystems USA). Sequencing results were viewed using FinchTV v1.4.0 software (Geospiza Inc.). Nucleotide sequences were interrogated using the BLAST (Basic Local Alignment Search Tool) algorithm at the National Center for Biotechnology Information (NCBI) website (www.NCBI.nlm.nih.gov) in July 2013, and phylogenetic trees were compiled by the Mega 5 alignment tool and CLUSTALW program with 500 bootstrapping value.

Extraction and amplification of community genomic DNA. Genomic DNA was extracted from biomass obtained from the surfaces of PU coupons and from compost using the Powersoil DNA isolation kit (MoBIO Laboratories, USA) according to the manufacturer's instructions. The concentration of eluted DNA was measured using a NanoDrop 1000 spectrophotometer, and samples were stored at −20°C until required.

Analysis of the fungal community by TRFLP analysis. For fungal community analysis by terminal restriction fragment length polymorphism (TRFLP) analysis, the fungal ITS1-5.8S-ITS2 rDNA region was amplified using the fluorescence-labeled primers, ITS5-FAM (6-carboxyfluorescein [FAM]-GGAAGTAAAAGTCGTAACAAGG) and ITS4-HEX (hexachloro-fluorescein [HEX]-TCCTCCGCTTATTGATATGC). A 50-μl PCR mix was made using 1 × NH_4 buffer, 1.5 mM $MgCl_2$, 200 μM each dNTPs, 0.5 U *Taq* polymerase (Bioline UK), 0.2 μM primers, and bovine serum albumin (BSA) (100 μg/ml) (New England BioLabs UK). Approximately 50 to 100 ng DNA template was used in each PCR mixture. The PCR regime consisted of an initial denaturation at 94°C for 10 min and then 35 cycles, with 1 cycle consisting of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min. PCR products were verified by gel electrophoresis, and three PCR amplicon replicate samples were pooled and purified by ethanol precipitation. Ethanol (100%) was added to the pooled DNA samples at a 2:1 ratio, and the resulting samples were incubated overnight at −20°C. The tubes were centrifuged at 13,000 rpm for 30 min at 0°C, and the supernatant was discarded. Ice-cold 70% (vol/vol) ethanol was added to the pellet and gently mixed by inverting the tubes several times, and then the tubes were centrifuged at 13,000 rpm at 0°C for 10 min. The supernatant was discarded from each tube, and the pellet was left to air dry overnight at room

temperature. The pellet was dissolved in 10 μl of sterile diethyl pyrocarbonate (DEPC)-treated water, and DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermofisher Scientific Inc., USA). To produce a mixture of end-labeled ITS rDNA fragments with variable lengths, PCR products (1.5 μg) were digested with 0.5 U HhaI (Fermentas, United Kingdom) at 37°C overnight in Tango buffer (10 μl; Fermentas, United Kingdom). Digested products (0.5 μl) were mixed with 9.25 μl Hi-Di formamide (ABI, United Kingdom) and 0.25 μl GS500LIZ (ABI, United Kingdom) in a 96-well PCR plate, and the products were separated and analyzed in-house on an ABI Prism 3100 genetic analyzer (Applied Biosystems USA) (27).

The size of the fragments was determined using Peak Scanner Software version 1.0 (Applied Biosystems), using peak height detection of 50 fluorescent units. The output was further analyzed using the online T-Align program (http://inismor.ucd.ie/~talign/) to generate a consensus profile of TRFs sizes between the technical duplicates and to compare the profiles between the samples (28). The Shannon-Weaver index ($\hat{H}$) and evenness (e) were measured for each TRFLP by the method of Tiquia (29). Principal component analysis (PCA) was employed to cluster the samples based on the relative intensity profile of TRFs using MVSP (multivariate statistical package) version 3.13g (Kovach Computing Services).

454 pyrosequencing. Fusion primers were designed with an adapter (lowercase) and key *TCAG* sequence with ITS5 and ITS4 primer sequences (uppercase) for unidirectional reads. The forward primers also had one of 10-bp unique Roche multiplex identifiers (MID) that were used to tag PCR amplicons from each sample. The sequence of the forward primer (primer A) was 5′-ccatctcatccctgcgtgtctccgac*TCAG*-(MID)-GGAAGTAAAAGTCGTAACAAGG-3′, and the sequence of the reverse primer (primer B) was 5′-cctatcccctgtgtgccttggcagtc*TCAG*TCCTCCGCT TATTGATATGC-3′. The 10 MID identifier sequences used were MID1 (ACGAGTGCGT), MID2 (ACGCTCGACA), MID3 (AGACGCACTC), MID4 (AGCACTGTAG), MID5 (ATCAGACACG), MID6 (ATATCGCG AG), MID7 (CGTGTCTCTA), MID8 (CTCGCGTGTC), MID10 (TCTC TATGCG), and MID11 (TGATACGTCT) (30).

PCR was conducted using the High Fidelity PCR system (Roche, USA). Fifty microliters of reaction mixture contained 1 × NH_4 buffer, 1.5 mM $MgCl_2$, 200 μM each dNTPs, 1 U polymerase, 0.2 μM primers (high-performance liquid chromatography [HPLC] purified), BSA (100 μg/ml) (New England BioLabs), 2 μl dimethyl sulfoxide (DMSO), and ca. 50 to 100 ng of DNA template per PCR mixture. The PCR regime used was similar to that used for TRFLP, except 30 cycles were used to reduce chimera formation. PCR products were verified by running the PCR mixture on a 1% (wt/vol) agarose gel (100 V for 45 min) containing 0.005% (wt/vol) ethidium bromide and visualizing the PCR products by using a UV transilluminator. The products were excised from the gel (ca. 575- to 700-bp expected size range) using a sterile rectangular blade, and DNA was extracted using a gel extraction kit (Qiagen, United Kingdom) according to the manufacturer's instructions. The products were further purified through column purification (Qiagen, United Kingdom) according to the manufacturer's instructions, and amplicons were pooled in equal concentration to give a final concentration of 10 ng/μl. Pooled samples were sent for 454 titanium platform pyrosequencing to the Centre for Genomic Research, University of Liverpool, United Kingdom.

Bioinformatics and statistical analysis. Sequence data processing was performed with MacQIIME version 1.6.0 following a procedure similar to that of Caporaso et al. (31). After splitting the libraries and denoising using default settings (32), sequences were grouped into operational taxonomic units (OTUs) at a similarity level of 97% using uclust (default) against the UNITE/QIIME 12_11 ITS reference database; any sequence that did not have a hit against the reference database were subsequently clustered *de novo*. A representative set of OTUs was generated, the taxonomy of each set was determined, and the OTUs were assigned using the UNITE and INSD databases. OTUs defined as 97% sequence similarity threshold were used for rarefaction curve, PCA, and beta diversity analysis (chao1 and Shannon-Weaver index) using QIIME.

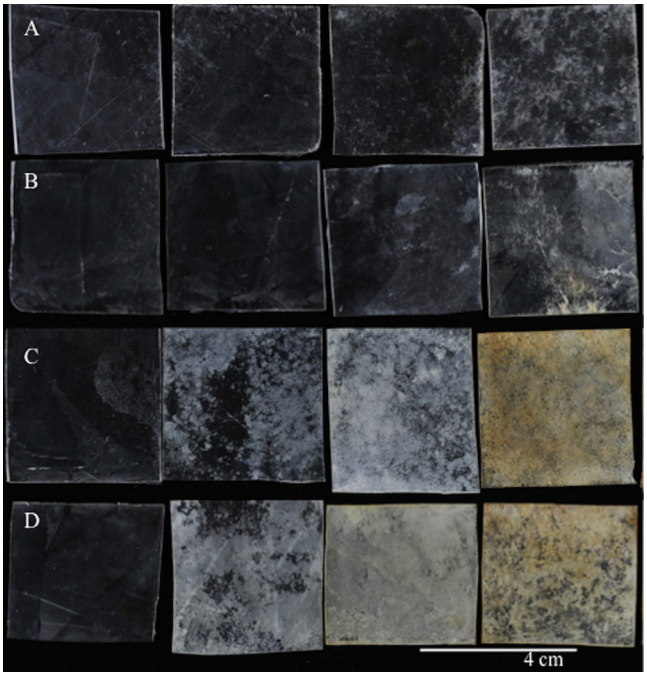


FIG 1 Visual changes to polyester polyurethane coupons after burial at different temperatures. Physical deterioration of polyester coupons buried in soil at 25°C (A) and buried in compost at 25°C (B), 45°C (C), and 50°C (D) for 0, 4, 8 and 12 weeks (shown from left to right). The white and cream discoloration visible on the surface is fungal mycelium.

Statistical analysis. To determine statistical significance, data were subjected to analysis of variance with the significance threshold set at a P value of 0.05 (JMP basic version 9.0.2; SAS Institute).

Nucleotide sequence accession numbers. The following accession numbers were obtained upon submission of the sequences: KF314698, JX996129, JX996130, JX996131, JX996132, JX996133, JX996134, JX996135, JX996136, JX996137, JX996139, and JX996140.

RESULTS

Moisture content and water-holding capacity. Moisture content of soils has previously been found to have a marked influence on fungal growth and degradation of PU (15). The water-holding capacity was determined to be 71% and 40% for compost and soil, respectively, and the moisture content was ca. 30% and 15% for compost and soil, respectively. Moisture content was maintained during the 12-week incubation period.

Visual changes to PU coupons during compost burial at different temperatures. To determine physical deterioration of PU coupons when buried in compost, PU coupons were buried and incubated at 25°C, 45°C, and 50°C. The coupons were recovered after 4, 8, and 12 weeks (Fig. 1). For comparative purposes, coupons were also buried in soil at 25°C. Initially, the PU coupons were transparent and flexible and had a smooth surface. After incubation at 25°C in soil or compost for 4 weeks, the surfaces of PU coupons appeared rough; visible cracks appeared after 8 weeks that increased in number and deepened after 12 weeks. White patches of mycelial growth were also clearly visible on the PU surface after 8 weeks and became extensive after 12 weeks (Fig. 1A and B). At 45 and 50°C, PU coupons became opaque after 4 weeks and fungal mycelium covered ca. 50 to 90% of the surface. Coupons were completely opaque after 8 weeks, and by the end of 12

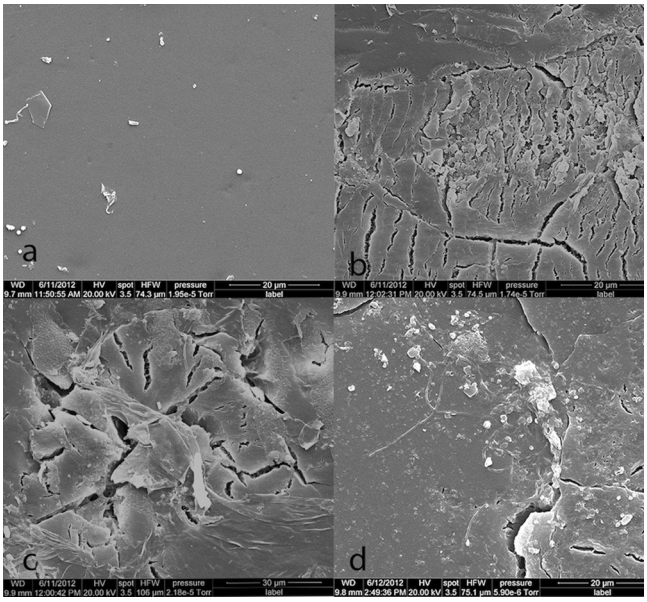


FIG 2 Effect of compost burial on the surface of PU coupons visualized by environmental scanning electron microscopy. (a to d) An unburied PU coupon (a) and PU coupons recovered after 12 weeks of burial in compost at 25°C (b), 45°C (c), and 50°C (d) and changes in the surface properties visualized by environmental electron microscopy. Prominent cracks can be seen on the surface of the PU.

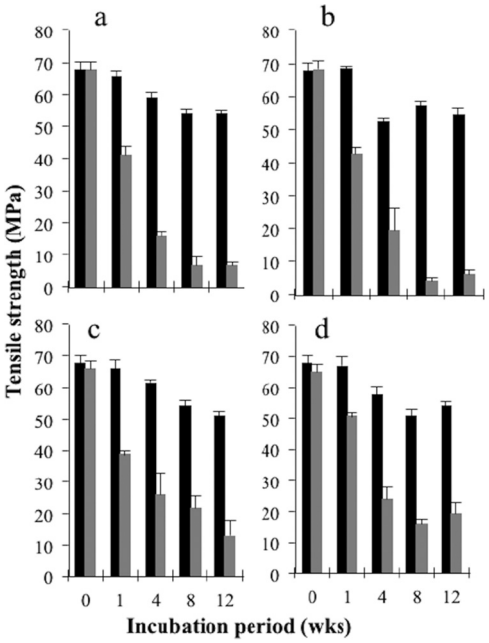


FIG 3 Effect of compost burial on the loss of tensile strength of PU coupons. PU coupons were buried in soil or compost, and loss of tensile strength was measured over a 12-week period. Control coupons were immersed in sterile soil or compost extract (black bars) and buried (gray bars) in soil at 25°C (a), compost at 25°C (b), compost at 45°C (c), or compost at 50°C (d). Values are means plus standard errors of the means (SEM) (error bars) of 5 replicates.

weeks, complete discoloration and surface cracking were observed (Fig. 1C and D).

To further examine the effects of burial on the surfaces of PU coupons, ESEM was conducted on PU coupons after 12 weeks of incubation (Fig. 2). Cracking of the PU surface was observed at all temperatures but was more extensive at 45 and 50°C. In contrast, no physical changes were observed in polyether PU coupons after 12 weeks burial at different temperatures (data not shown).

Impact of soil and compost burial on the tensile strength of PU. In order to quantify the extent of biodegradation during burial, the tensile strength of buried PU dumbbells was determined periodically following incubation at 25, 45, and 50°C for 12 weeks (Fig. 3a to d). In order to distinguish between microbiological effects and potential effects of chemical components in soil and compost, PU dumbbells were also immersed in sterile soil and compost extracts. The tensile strength of PU dumbbells buried in compost or soil at all temperatures after 12 weeks showed major losses ($P < 0.05$) in tensile strength (>75%) compared to dumbbells immersed in sterile soil or compost extract. There was slight variation ($P > 0.05$) in tensile strength loss at different temperatures; however, the variation in loss of tensile strength among replicate coupons increased with increase in temperature (data not shown). No considerable physical degradation was seen in polyether PU dumbbells after 12 weeks under any condition (data not shown).

To further assess biodegradation, dry weight loss was determined after 12 weeks of burial. Despite the major reduction in tensile strength and physical disruption of the surface of PU, weight loss in all cases was <1% and was not significant ($P > 0.05$) (data not shown).

Fungal colonization of PU coupons. To investigate fungal colonization of PU coupons following burial, total fungal viable counts and putative fungal PU degrader counts recovered from the surfaces of coupons were enumerated on compost extract agar (CEA) (15) and PUA plates, respectively (Fig. 4A). Total fungal viable and putative fungal degrader counts in the compost and soil environments in which the PU coupons were buried were also enumerated (Fig. 4B). All fungal colonies recovered on PUA agar displayed a zone of clearing around the colony margin indicating PU degradation.

Total viable counts in soil and in compost in which the coupons were buried remained similar for any temperature over 12 weeks. At 25°C, total viable counts in soil and compost were similar; however, counts in compost were significantly ($P < 0.05$) lower at 45°C and 50°C, temperatures at which only thermotolerant and thermophilic fungi can grow (33). When total viable counts and putative PU degrader counts were compared, PU degraders composed ca. 40 to 70% of all colonies recovered (Fig. 4B), indicating that a large proportion of the viable fungal population was putative degraders.

Total fungal viable counts from the surfaces of PU coupons buried in soil and compost at 25°C increased up to week 8 and then remained similar. Total viable counts from the PU surface also increased up to week 8 when incubated at 45°C and 50°C but decreased at week 12. A comparison of total viable counts and putative degrader counts demonstrated that ca. >70% of fungi colonizing the PU surface were putative degraders (Fig. 4A).

Identification of polyester PU-degrading fungal isolates. Fungal colonies growing on PUA plates were difficult to distinguish morphologically. Therefore, colonies were transferred onto PDA and incubated for up to 5 days. Differences in development of pigments, sporulation, and colonial morphology on PDA en-

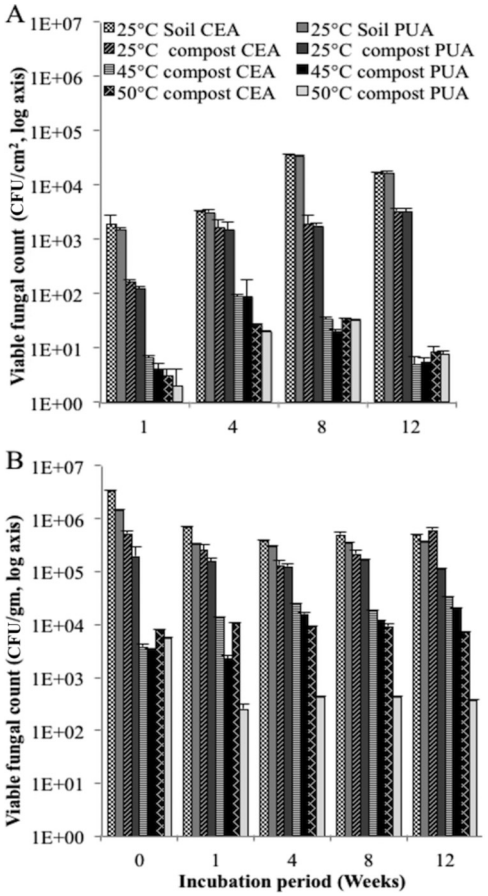


FIG 4 Changes in the total fungal viable counts and total fungal PU degrader counts over a 12-week incubation period. (A) Total viable and putative PU degrading fungal counts (CFU/cm^2) recovered from the surfaces of polyester PU coupons. (B) Total viable and putative PU-degrading fungal counts in compost/soil (CFU/g of compost/soil). Values are means plus SEM of three replicates.

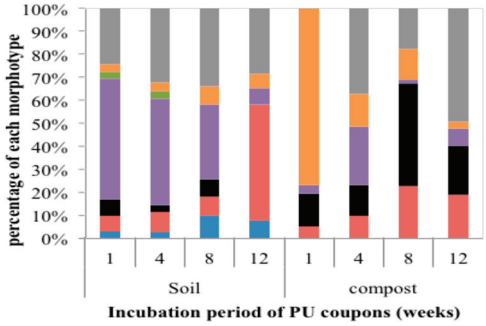


FIG 5 Relative distribution of the colony morphotypes recovered from the surfaces of PU coupons buried over 12 weeks at 25°C. Polyester PU coupons were buried in soil at 25°C and in compost at 25, 45, and 50°C for 12 weeks, and viable fungal colonies were recovered periodically from the surfaces of the coupons on PUA. Following transfer onto PDA, the colonies were classified into different morphotypes, and the percentage of fungal colonies recovered for each fungal morphotype was calculated. A total of seven major morphotypes were recovered. Morphotype 1 (*Alternaria* sp.) (blue), 2 (*Bionectria ochroleuca*) (red), 3 (*Geomyces pannorum*) (black), 4 (*Trichosporon moniliforme*) (purple), 5 (*Volutella ciliata*) (light green), 6 (*Penicillium* spp.) (pumpkin), 7 (*Fusarium* spp.) (gray). At 45°C and 50°C, only *Aspergillus fumigatus* and *Thermomyces lanuginosus*, respectively, were recovered.

abled colonies to be grouped into different morphotypes and quantified (Fig. 5). Four or five isolates were randomly chosen, total genomic DNA was extracted, the ITS1-5.8S-ITS2 region of rDNA was amplified by PCR and sequenced, and the sequences were used to interrogate the NCBI database (Table 1). All the isolates were then aligned with the published sequences, and a phylogenetic tree was constructed (Fig. 6). Although the two environments were very different (soil and compost), a number of common species were isolated at 25°C from the surface of PU from both soil and compost with only an *Alternaria* sp. (morphotype 1) and *Volutella ciliata* (morphotype 5) uniquely recovered from the surface of soil-buried PU. However, the relative proportions of the species recovered from the surface of PU differed between soil and compost and changed over the 12-week incubation period (Fig. 5). From the surface of soil-buried PU at 25°C, the proportion of the different species recovered was relatively constant except that *Volutella ciliata* could not be recovered after week 4, *Geomyces pannorum* could not be recovered after week 8 and *Bionectria ochroleuca* was the dominant species by week 12. The proportion of species recovered from the surface of PU buried in compost was also variable over the 12-week incubation period, *Penicillium* spp. were dominant at week 1, but from weeks 4 to 12, *Fusarium solani/Fusarium oxysporum* was the dominant phenotype. At 45 and 50°C, *Aspergillus fumigatus* and *Thermomyces lanuginosus*, respectively, were the only species recovered at any time point. For morphotypes 6 and 7, subsequent sequencing of random isolates revealed that they were composed of more than one species (*Penicillium mandriti*, *Penicillium chrysogenum*, and *Penicillium roseopurpureum* for morphotype 6 and *Fusarium solani* and *Fusarium oxysporum* for morphotype 7) (Table 1).

Fungal community diversity on the surface of buried polyester PU. TRFLP was used to study the temporal change in fungal communities on the surface of polyester PU during burial in soil at 25°C and in compost at 25, 45, and 50°C. TRF electropherograms were compared using principal component analysis (PCA) and displayed as a scatter graph for each separate temperature for both the communities on the PU surface and in the surrounding compost/soil (Fig. 7). At 25°C, the fungal communities in soil and in compost separated into two distinct groups (soil and compost [group I and III]), indicating two distinctive communities (Fig. 7A). Within soil and compost at all temperatures, the community changed over the first 4 weeks and then remained stable. However, the communities colonizing the PU surface were separate from the compost community, indicating that the community on the PU surface was different from the surrounding soil or compost. The community on the surface clustered together but was not stable and changed over the 12-week period. However, at 50°C, the clustering was much weaker than at 25 and 45°C on the PU surface, suggesting greater community change over time at the higher temperature (Fig. 7A, B, and C).

TRFLP profiles generated from compost, soil, and PU coupons were subjected to statistical analysis using MVSP and the total number of TRFs and their relative abundance was used to calculate Shannon-Weaver index and evenness as indicators of diversity and equitability, respectively (Table 2). When soil or compost was incubated at 25°C, the Shannon-Weaver index increased from week 0 to week 4 and then remained approximately constant, indicating an initial increase in species diversity. The Shannon-

TABLE 1 Description, identification, and frequency of occurrence of fungal isolates of different morphotypes recovered from surfaces of buried polyester PU coupons

NCBI accession no.	Morphotype	Closest match in NCBI database	Frequency on PUA plates[a]	Temp. (°C) and environment[b]	Homology (%)
KF314698	Brown colored, septate (morphotype 5)	*Volutella ciliata*	++	25 s	100
JX996129	Yellowish cottony mycelium (morphotype 7)	*Fusarium solani*	+++	25 c+s	100
JX996130	Olive green, filamentous	*Aspergillus fumigatus*	+++	45+50 c	100
JX996131	Yellowish cottony mycelium (morphotype 7)	*Fusarium oxysporum*	+++	25 c+s	100
JX996132	Yeast like (morphotype 4)	*Trichosporon moniliforme*	+++	25 c+s	99
JX996133	Dark green color, granular (morphotype 6)	*Penicillium mandriti*	++	25 c	99
JX996134	Dark green color, granular (morphotype 6)	*Penicillium chrysogenum*	++	25 c+s	99
JX996135	White cottony (morphotype 2)	*Bionectria ochroleuca*	++	25 c+s	99
JX996136	Dark green color, granular (morphotype 6)	*Penicillium roseopurpureum*	+	25 c	99
JX996137	Gray (morphotype 1)	*Alternaria* sp.	++	25 s	98
JX996139	Brown, floccose, radial edges (morphotype 3)	*Geomyces pannorum*	++	25 c+s	100
JX996140	Peach, felt like	*Thermomyces lanuginosus*	+++	50 c	100

[a] Symbols: +++, dominant; ++, frequent; +, occasional.
[b] Abbreviations: c, compost; s, soil.

Weaver index from the surface of PU remained approximately constant over 12 weeks but was significantly lower ($P < 0.05$), indicating that the community on the surface was far less diverse than the surrounding compost or soil. At 45 and 50°C, the Shannon-Weaver index decreased at week 4 and then remained approximately constant, indicating selection for thermophilic and thermotolerant species and a consequent reduction in species diversity. Again, the Shannon-Weaver index for the PU surface was significantly ($P < 0.05$) lower than that for the surrounding compost (Table 2). Evenness remained approximately constant in soil and compost and on the surface of PU at all temperatures, indicating no emergence of dominant species in the community over time; however, the evenness value from the PU surface was lower than the surrounding soil or compost, indicating a less even distribution in the surface community.

Biodiversity of fungal communities by pyrosequencing. A total of 94,502 sequences were obtained after discarding low-quality and short reads. OTUs defined at 97% sequence similarity threshold were used to generate the rarefaction curve. The curve leveled off in almost all samples, indicating that the libraries provided an adequate sampling of fungal diversity. The number of observed species ranged between 14 and 51, with the highest number detected at 25°C (Fig. 8, top). The chao1 index, an estimator of OTU richness, was highest at 25°C in the compost and lowest in the 50°C compost, while no major difference was seen on the surface of PU coupons among the three different temperatures (Table 3). In compost at 25°C, *Geomyces pannorum* was the dominant species prior to incubation and after 12 weeks (49.6% and 42.2%, respectively), while other species declined or were not detected (*Thielavia* sp., *Arthrographis kalrae*, *Pseudallescheria boydii*, *Arthrobotrys flagran*, and *Doratomyces nanus*). After 12 weeks, other species appeared at levels of >1% that were not detected or present at less than 1% prior to incubation (*Scytalidium thermophilum*, *Fusarium solani*, *Mortierella* sp., *Pseudallescheria fimeti*, and *Thermomyces lanuginosus*). Incubation of compost at 45°C and 50°C for 12 weeks led to the development of a very different community compared to compost prior to incubation due to the selection of thermophilic and thermotolerant species. Three species, *Emericella rugulosa*, an unidentified fungal clone A, and *Scytalidium thermophilum*, were present at both 45°C and 50°C and accounted for 81% and 88.9% of all sequences, respectively, although *Scytalidium thermophilum* was predominant at 45°C and *Emericella rugulosa* at 50°C (Fig. 9).

Pyrosequencing revealed clear differences in the fungal communities in the compost compared to those from the surfaces of the PU coupons at each temperature (Fig. 9). At 25°C, the population of *Geomyces pannorum* in compost was 42.4%, but on the surfaces of PU coupons, it was present at <1% with *Fusarium solani* dominating (53%). At 45°C and 50°C, the fungal communities on the surfaces of PU coupons were similar to each other and dominated by *Candida ethanolica* (60.3% and 57.6%, respectively), an unidentified fungal clone A (32.3% and 29.0%, respectively), and *Penicillium paneum* (2.4% and 2.5%, respectively). *Candida rugosa* (4.9%) was the only species present at >1% that was found only at 50°C. The compost community at 45°C was dominated by *Scytalidium thermophilum* (68.1%), whereas at 50°C, *Emericella rugulosa* was dominant (60.8%). PCA analysis of the OTUs also demonstrated that the compost community at 45°C and 50°C clustered together, and a second distinct cluster was composed of communities on the surface of PU at 45 and 50°C (Fig. 8, bottom). PCA analysis also showed that incubation of compost at 45 and 50°C had a greater effect on the fungal community than incubating at 25°C, but nonetheless, the community on the surface of PU at 25°C was different from that of the surrounding compost.

DISCUSSION

Polyurethanes are a diverse group of plastics with a wide range of applications ranging from packaging materials, shoe soles, coatings, and paints and are therefore common environmental pollutants (2, 34). Polyester PUs are known to be susceptible to microbial enzymatic degradation through hydrolysis of the polymer (20), and it has been shown previously that fungi are the dominant microbes responsible for colonization and biodegradation of PUs in soil environments, particularly when the moisture content falls between 20 and 70% (15–17).

Composting is a natural process involving the aerobic decomposition of organic wastes by a mixed microbial consortium that also involves thermophilic microbes appearing due to the increase in heat that occurs during microbial respiration and in recent years has been increasingly used commercially as a waste management treatment for green waste and food waste (9). In this study,

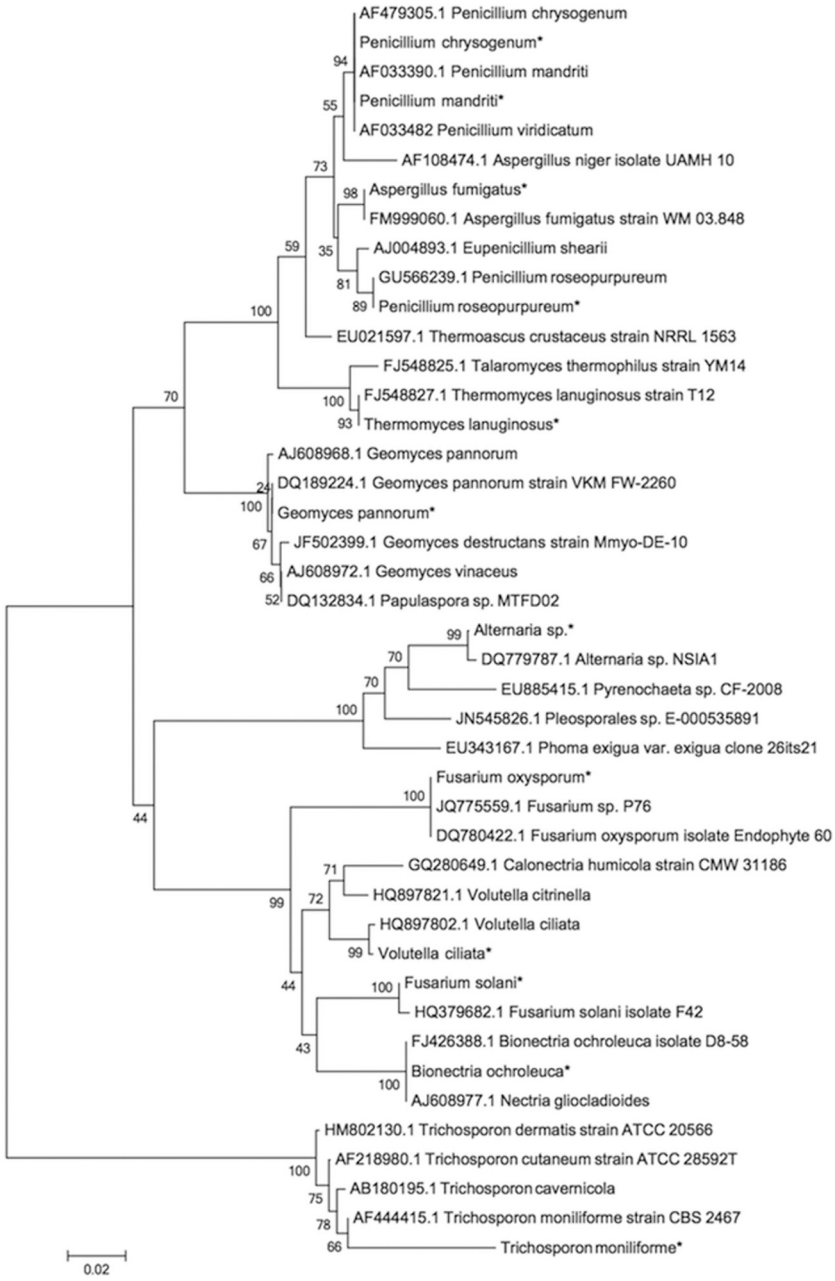


FIG 6 Neighbor-joining phylogenetic analysis of putative isolated PU-degrading fungi. Fungi isolated and identified from the surfaces of PU coupons (Table 1) (indicated by an asterisk) were subjected to phylogenetic analysis with closely related species using neighbor-joining phylogenetic tree (bootstrap corrected with 500 samples). Sequences were interrogated by the BLAST algorithm in July 2013. The numbers at the nodes indicate bootstrap values (as percentages of 500 replicates), and the scale bar indicates branch length.

we investigated whether microbes, and in particular fungi present in compost, had the capacity to degrade PU and whether selection of particular communities on the surfaces of PU coupons were associated with this process. For this purpose, we incubated PU coupons for up to 12 weeks in mature compost at 25°C (to emulate the maturation phase) and at 45°C and 50°C (to emulate the thermophilic stage). Coupons were also buried in soil at 25°C to enable the rate of degradation in soil to be compared to that in compost. Significant colonization by fungal mycelia was clearly observed in all cases along with significant macroscopic changes to the PU coupons (Fig. 1), and severe cracking of the PU surface and fungal hyphae were visible by environmental scanning electron microscopy (Fig. 2). Barratt et al. (15) also observed a high coverage of polyurethane film by fungal hyphae and spores and extensive surface damage in soil-buried polyester PU. Despite these major changes to the integrity of the coupons, little loss in dry weight was observed. Weight loss over 12 weeks was not therefore a reliable indicator for polyester PU biodegradation. Barratt et al. (15) and Pathirana and Seal (20) also reported little change in weight loss in soil-buried PU.

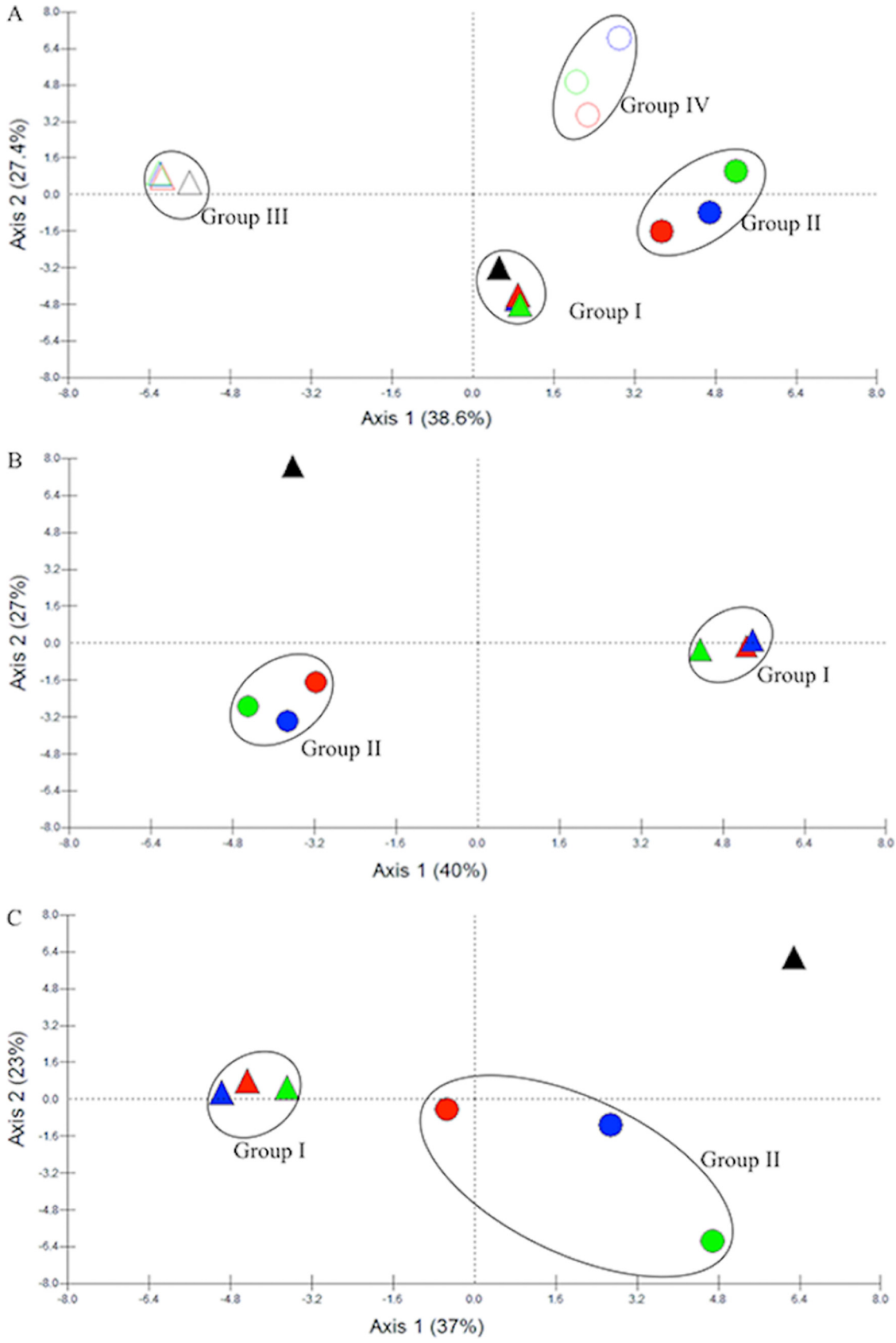


FIG 7 Principal component analysis of microbial communities obtained from the surface of PU and compost. (A to C) Scatter plot for principal component analysis of TRFLP pattern obtained from the surface of the buried polyester PU coupon and compost/soil at 25°C (A), 45°C (B), and 50°C (C) after week 4 (red), 8 (green), and 12 (blue). Fungal community in compost/soil and on the surface of PU showed obvious demarcation. PU surface buried in compost at week 4 (solid red circle), week 8 (solid green circle), and week 12 (solid blue circle), compost at week 0 (solid black triangle), week 4 (solid red triangle), week 8 (solid green triangle), and week 12 (solid blue triangle), PU surface buried in soil at week 4 (red-outlined circle), week 8 (green-outlined circle), and week 12 (blue-outlined circle), and soil at week 0 (black-outlined triangle), week 4 (red-outlined triangle), week 8 (green-outlined triangle) and week 12 (blue-outlined triangle).

TABLE 2 Variation in the Shannon-Weaver and evenness indices derived from TRFLP electropherograms from fungal communities

Sample type, temp, and incubation time	Shannon-Weaver index[a]		Evenness[a]	
	Compost or soil	PU surface	Compost or soil	PU surface
Soil 25°C				
0 wk	2.9	n/a	0.7	n/a
4 wk	3.4	2.5	0.7	0.6
8 wk	3.7	2.7	0.8	0.6
12 wk	3.2	2.4	0.8	0.7
Compost 25°C				
0 wk	2.8	n/a	0.8	n/a
4 wk	3.5	2.6	0.8	0.7
8 wk	3.3	2.5	0.8	0.7
12 wk	3.4	2.5	0.8	0.8
Compost 45°C				
0 wk	2.8	n/a	0.7	n/a
4 wk	2.2	1.4	0.8	0.6
8 wk	1.9	1.1	0.7	0.7
12 wk	2.1	1.5	0.6	0.6
Compost 50°C				
0 wk	2.9	n/a	0.7	n/a
4 wk	2.0	1.1	0.6	0.6
8 wk	2.0	1.8	0.7	0.6
12 wk	2.1	1.7	0.7	0.7

[a] n/a, not available (no fungi were detected from the PU surface prior to burial).

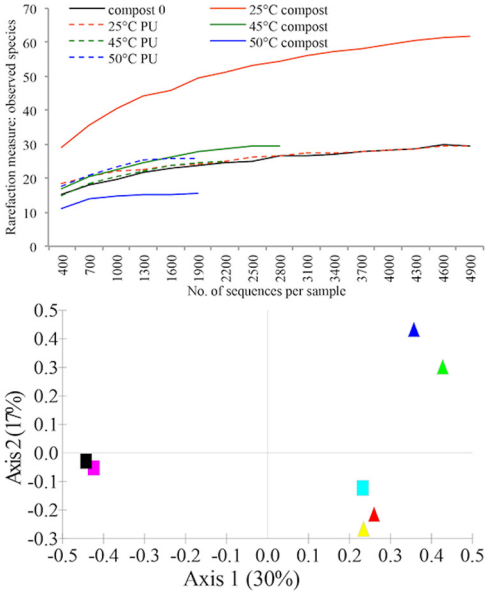


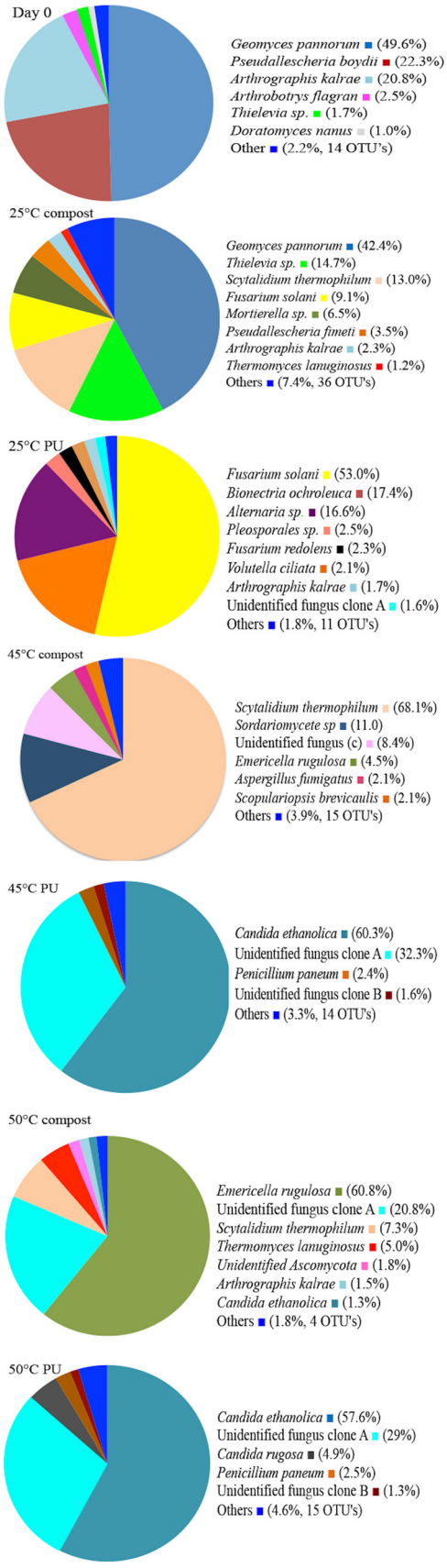
FIG 8 Calculated rarefaction curves of observed species and principal component analysis based on pyrosequencing data. (Top) Rarefaction curve suggested library saturation at 5,000 sequences. (Bottom) Principal component analysis suggested distinct communities in compost and surfaces of PU coupons. Compost at week 0 (navy blue triangle), compost and PU coupons after week 12 at 25°C (green triangle and blue-green square, respectively), compost and PU coupons after week 12 at 45°C (red triangle and fuchsia square, respectively), compost and PU coupons after week 12 at 50°C (yellow triangle and black square, respectively).

Significant changes were observed in the tensile strength of buried polyester PU (Fig. 3). Tensile strength is defined as the load at break divided by the original cross-sectional area. A number of studies have analyzed the rate of degradation of PU by analyzing loss in tensile strength, as it is a sensitive measure of polymer integrity (15–18, 35–38). Umare and Chandre (39) studied the degradation of polyester PU under three different treatments, alkaline hydrolysis, enzymatic hydrolysis, and soil burial, and reported that soil burial gave the greatest level of degradation. Previously Barratt et al. (15) buried polyurethane in soil microcosms and reported a reduction in tensile strength of 60% after 44 days. In another study, Cosgrove et al. (17) investigated the rate of degradation of polyester PU *in situ* in the environment and found a ca. 95% reduction in tensile strength after 5 months. In our study, the values for percent loss in tensile strength of buried polyester PU coupons at 25, 45, and 50°C in compost were ca. 90, 80, and 70, respectively, suggesting that in compost, PU degradation occurs to a similar extent at all phases of the composting process, albeit by different fungal species. Loss in tensile strength has been shown to be due to the secretion of extracellular enzymes that degrade the ester and urethane linkages of polyurethane causing polymer chain scission (20, 40, 41). A fall in tensile strength was also observed when polyester PU coupons were incubated for the same length of time in sterile compost extract, although to a much lower extent (Fig. 3). Aquino et al. (42) studied hydrolysis of polyester PU over a wide temperature range (10 to 70°C) and found that PU was stable at 50°C, but significant polymer hydrolysis occurred at 70°C after 177 days of incubation. In another study, Zuidema et al. (43) also reported significant hydrolysis of PU at two different temperatures (37°C and 60°C) after 3 years. Thus, while polyester PU is vulnerable to chemical abiotic hydrolysis, it is a slow process and does not account for the large drop in tensile strength observed when buried in soil or compost.

In contrast, no change in tensile strength was observed after 3 months burial under any condition for polyether PU (data not shown). Polyether PU is known to be far more recalcitrant to degradation than polyester PU is (3, 44). Darby and Kaplan (7) also compared the biodegradation of polyether- and polyester-based PU and suggested that polyether PUs are moderately to highly resistant to fungal attack than polyester PUs because of the

TABLE 3 Statistical evaluation of fungal communities from compost and from the surfaces of PU coupons buried in compost incubated at different temperatures for 12 weeks

Sample and temp	Shannon-Weaver index	Chao1 index
Compost initial (ambient) (time zero) (control)	2.3	30.9
25°C		
Compost	3.0	63.2
PU surface	2.7	29.4
45°C		
Compost	1.8	32.1
PU surface	1.8	27.1
50°C		
Compost	1.0	15.5
PU surface	1.8	29.7



presence of high-molecular-weight branched polyol chains. A similar observation was made by Filip (45), when PU biodegradation was tested in landfills. Jansen et al. (46) reported that polyether PU degradation occurred very slowly when *Staphylococcus epidermis* was used as a test organism, and polyether-based PU was also found to be highly resistant to anaerobic bacterial degradation. Krasowska et al. (3) reported that polyether-based PU turned yellow/brown when incubated in a compost pile but reported no physical deterioration. However, Matsumiya et al. (47) reported that an *Alternaria* sp. could degrade polyether polyurethane physically, and released metabolites were detected in the medium, indicating physical degradation. This appeared to be due to the hydrolysis of urea and urethane bonds releasing polyols and polyisocyanates. In our study, polyether PU was incubated for 12 weeks and may require a much longer incubation period before a significant change in tensile strength is observed.

TRFLP has previously been successfully used to analyze species diversity in fungal communities from a wide variety of environments (48–50). PCA analysis of TRFLP electropherograms also demonstrated clear differences in the communities colonizing the surface of PU compared to the surrounding environment, indicating selection on the PU surface (Fig. 7). Previously, Cosgrove et al. (17) also reported the polyester PU buried in two soil types using denaturing gradient gel electrophoresis (DGGE) showed different fungal communities on the surface of PU compared to the surrounding compost.

The fungal species isolated and cultured from the surface of polyester PU at 25°C in both soil and compost after 12 weeks were the same (*Bionectria ochroleuca*, *Fusarium oxysporum*/*Fusarium solani*, and *Trichosporon moniliforme*) although recovered to different extents (Fig. 5). The only exception was *Geomyces pannorum*, which was recovered from the PU surface in compost, but not in soil, although it was recovered at weeks 1, 4, and 8. Many of the fungi recovered have been reported to be associated with PU degradation previously (8, 15, 17, 20, 22, 35). Thus, culturing suggests a limited species diversity on the surface of PU when buried in either soil or compost, but cultivation is known to be unreliable and to underreport the true diversity of microbial populations, as only a highly limited proportion of species (estimated to be <1 to 5%) grow readily on synthetic media (51, 52).

In order to examine the diversity of the fungal communities on the surfaces of PU coupons buried in compost in more detail, pyrosequencing was performed with total genomic DNA extracted from mycelium colonizing the surfaces of the PU coupons after 12 weeks of burial at 25, 45, and 50°C and compared to the surrounding compost fungal community (Fig. 8 and 9). 454 pyrosequencing is a high-throughput sequencing technique particularly useful in the detection of rare community members that may represent <1% of the microbiota (53). Recently, 454 pyrosequencing demonstrated that fungal diversity is rich in soil with detection of rare OTUs that were previously underestimated (54,

FIG 9 454 pyrosequencing data obtained for fungal community on week 0 and 12 from compost and the surfaces of PU coupons at 25, 45, and 50°C. The fungal community on the surfaces of PU coupons is different from that in compost, and change was observed in compost from weeks 0 to 12. At 25°C, *Fusarium solani* was the dominant species, and at 45 and 50°C, *Candida ethanolica* was the dominant species. There were different clusters of sequences assigned as the unidentified fungal clone, which are differentiated as clone A and clone B.

55). The results of PCA analysis of the pyrosequencing data were broadly congruent with the results of PCA analysis of the TRFLP electropherograms in demonstrating that the fungal community on the surface of PU was different from the surrounding compost community at each temperature and that the PU surface community at 45 and 50°C were broadly similar (Fig. 8, bottom).

On the surface of PU at 25°C, *Fusarium solani*, *Bionectria ochroleuca*, and an *Alternaria* sp. accounted for 53.0%, 17.4%, and 16.6% of the sequences recovered. After 12 weeks, both *F. solani* and *B. ochroleuca* were also the dominant organisms recovered by cultivation (Fig. 5). *F. solani* has previously been associated with the degradation of polyester PU (22, 35), and *B. ochroleuca* was also identified as a dominant organism from the surface of polyester PU buried in soil after pretreatment with impranil DLN (a polyester PU dispersion) following cloning and sequencing of a major band found by DGGE (16). Interestingly, it was also isolated from soil as a major degrader of poly(butylene succinate) films, a biodegradable polyester polymer, suggesting that it possesses the ability to efficiently degrade esters within long-chain polymers (56). *Alternaria* spp. have also been ubiquitously found in soil and compost, and several *Alternaria* species have been extensively reported as polyurethane degraders (8, 17, 19, 37, 47, 57).

While the most dominant species cultivated from the surface of PU at 25°C were among the most dominant species identified by pyrosequencing, at 45°C and 50°C, there was little correlation. At 45° and 50°C, *A. fumigatus* and *T. lanuginosus* were the only fungi isolated via culture-based technique (Table 1). However, pyrosequencing revealed that in both cases, *Candida ethanolica* was the dominant organism on the surface of PU. *C. ethanolica* was first isolated as an ethanol-tolerant yeast with a limited ability to ferment sugars and is closely related to the *Pichia* group (58). It has, however, been reported to be among a group of yeasts identified from a clone library which dominated the early stages of composting in a commercial compost facility (59). Also dominant was an unidentified fungal clone A that was present at both 45°C and 50°C and that has been found in soils but has yet to be isolated or taxonomically assigned. The third most dominant species was *Candida rugosa* comprising 4.9% of the population on the surfaces of PU coupons at 50°C. Gautam et al. (60) have demonstrated that a lipase enzyme from *C. rugosa* was able to cause significant PU degradation. *Penicillium paneum* was also detected on the surfaces of PU coupons and is closely related to *Penicillium roqueforti* and has been reported to grow under highly acidic environments (61). Together, *C. ethanolica* and unidentified fungus clone A accounted for >85% of the sequences recovered, with the remaining sequences belonging to 14 to 16 other species (Fig. 9). While *A. fumigatus* and *T. lanuginosus* were detected by pyrosequencing on the surface of PU, they accounted for <0.35% of the sequences, despite appearing to be the only organisms isolated from the surface of PU through conventional cultivation.

In summary, this study has demonstrated that polyester PU is susceptible to fungal biodegradation in compost under both thermophilic (thermophilic stage) and mesophilic (maturation phase) conditions and that positive selection for rare taxa from the existing compost community on the PU surface occurs. Thus, there may be the potential for optimizing existing commercial composting processes to enable polyester PU waste to be diverted into commercial composting streams, thereby reducing the burden on landfill sites.

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